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I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application, Provisional & Complete Specification*** *and* ***Drawing Sheets*** *filed in connection with Application for Patent No.* ***1598/Del/2003 dated 23rd December 2003.***

Witness my hand this 7th day of February 2005.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

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1598-DEL-03

FORM 1

23 DEC 2003

THE PATENTS ACT, 1970
(39 of 1970)
APPLICATION FOR GRANT OF A PATENT
(See sections 5(2), 7, 54 and 135 and rule 39)

1. I/We, All India Institute of Medical Sciences, Div of Clinical Microbiology, Dept. of Laboratory Medicine, Anari Nagar, New Delhi -110 0291, India and **DEPARTMENT OF BIOTECHNOLOGY**, a Dept. of Govt. of India, CGO Complex, Lodhi Road, New Delhi 110 003

2. hereby declare -
   (a) that I am/we are in possession of an invention titled: **"Oligonucleotides for detection of Leishmaniasis and methods thereof"**
   (b) that the **Provisional Specification** relating to this invention is filed with this application.
   (c) that there is no lawful ground of objection to the grant of a patent to me/us.

3. further declare that the inventor(s) for the said invention is / are :

   Singh, Sarman
   Dept. of Laboratory Medicine, Div of Clinical Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110 029; Indian Citizen,

4. I/We, claim the priority from the application(s) filed in convention countries. particulars of which are as follows: Nil

5. I./We, state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which I/We are the applicant/patentee : **NA**

6. I/We, state that the application is divided out of my/our application, the particulars of which are given below: **Nil**
   Application No.: **Nil** and pray that this application deemed to have been filed on **NA** under section 16 of the Act.

7. That I am/We are the assignee or legal representative of the true and first inventors.

8. That my/our address for service in India is as follows:

   LAKSHMIKUMARAN & SRIDHARAN
   B4/158, SAFDARJANG ENCLAVE,

NEW DELHI 110 029, INDIA
Tel: 011- 2619 2243/73/80 Fax: 2619 7578

9. Following declaration was given by the inventor(s) or applicant(s) in the convention country:

I/We the true and first inventors for this invention of or the applicant(s) in the convention country declare that the applicant(s) herein is/are my/our assignee or legal representative.

Singh, Sarman	Dept. of Laboratory Medicine, Div of Clinical Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110 029; Indian Citizen.	
Name of first inventor	Address and Nationality	Signature

10. That to the best of my /our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me/us on this application

11. Following are the attachment with the application:
(f) ~~Complete~~ Provisional specification in duplicate
(g) Drawings in duplicate
(h) Statement and undertaking on FORM-3 in duplicate
(i) Power of Attorney (To follow)
(j) Form 5

I/We request that a patent may be granted to me/us for the said invention.

Dated this 23 day of December, 2003

V.Lakshmikumaran
Attorney for the Applicant

To
The Controller of Patents
The Patent Office, at Delhi

1598DEL07

23 DEC

FORM 2

THE PATENTS ACT, 1970
(39 of 1970)

PROVISIONAL SPECIFICATION
(See section 10)

"Oligonucleotides for Detection of Leishmaniasis and methods thereof"

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, Divisional of Clinical Microbiology, Dept of Laboratory Medicine, Ansari Nagar, New Delhi -110 029, India and **DEPARTMENT OF BIOTECHNOLOGY**, a Dept. of Govt. of India, CGO Complex, Lodhi Road, New Delhi 110 003

The following specification describes the nature of the invention which it is to be performed.

Oligonucleotides for Detection of Leishmaniasis and methods thereof

FIELD OF THE INVENTION

The present invention provides a method to detect Leishmaniasis by PCR amplification of the conserved immunodominant tandem repeat region of Kinesin gene from various strains of *L. donavani.* This invention also provides unique primers and a PCR method using these primers to differentiate visceral leishmaniasis from post kala-azar-dermal leishmaniasis.

BACKGROUND/ PRIOR ART

Leishmaniasis, a vector-borne parasitic disease, is caused by obligate intramacrophage protozoa. It is characterized by diversity and complexity. It presents itself with a wide range of clinical forms. However, there are mainly 4 clinical forms. The Visceral Leishmaniasis (VL), also known as *kala azar*, is the most severe form of the disease, which, if untreated, has a mortality rate of almost 100%. The Cutaneous Leishmaniasis (CL) produces skin ulcers on the exposed parts of the body, such as the face, arms and legs. The number of ulcers may vary from 1 to as many as 200 in some cases, causing serious disability and leaving the patient permanently scarred. The third form is Mucocutaneous Leishmaniasis (MCL), or *espundia.* It can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities and can involve even the cartilage. The cutaneous form may lead to disseminated form, known as Diffuse Cutaneous Leishmaniasis (DCL). Leishmaniases are caused by a total of about 21 species, which are transmitted by about 30 species of *Phlebotomine* sandflies [Herwaldt BL., 1999].

The leishmaniasis are presently endemic in 88 countries on five continents, Africa, Asia, Europe, North America and South America, and a total of 350 million people are at risk of infection. It is estimated that worldwide 12 million people are affected by leishmaniasis; this figure includes cases with overt disease and those with no apparent symptoms. Of the 1.5-2 million new cases estimated to occur annually, only 600 000 are officially declared. Of the 500 000 new cases of VL, which occur annually, 90%, are in five developing countries: Bangladesh, Brazil, India, Nepal and Sudan. About 90% of all cases of MCL occur in Bolivia, Brazil and Peru and 90% of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually worldwide. The geographical distribution of leishmaniasis

is limited by the distribution of the sandfly, its susceptibility to cold climates, its tendency to take blood from humans or animals only and its capacity to support the internal development of specific species of *Leishmania* [Desjeux P 2001].

Since 1993, regions that are *Leishmania*-endemic have expanded significantly, accompanied by a sharp increase in the number of recorded cases of the disease. The geographic spread is due to factors related mostly to development. These include massive rural-urban migration and agro-industrial projects that bring non-immune urban dwellers into endemic rural areas. Man-made projects with environmental impact, like dams, irrigation systems and wells, and deforestation contribute to the spread of leishmaniasis. AIDS and other immunosuppressive conditions increase the risk of *Leishmania*-infected people developing visceral illness [Desjeux P 2001, Paredes R *et al.,* 1997].

VL is primarily caused by *L. donovani* in the Indian subcontinent and Africa, *Leishmania infantum* in Mediterranean region and *Leishmania chagasi* in the new world; of these species *Leishmania chagasi* and *Leishmania infantum* are closely related. Although, all the above species cause VL they are genetically different from each other. The data obtained by Cupolillo *et al.,* [1994] using numerical zymotaxonomy showed that *L. chagasi*, the new world visceralising species is similar to the old world *L. infantum*. The Zymodeme, serodeme, quantitative comparisons of nuclear DNA fragment patterns all indicates that *L. chagasi* and *L. infantum* are closely related and may represent the same species. Also, the study by Beverley S.M *et al.,* [1987] based on nuclear DNA restriction fragment patterns reveals that, the *L. chagasi* and *L. infantum* are similar and as closely related to each other as two random individuals from the same population and *L. donovani* is different from these two species. In another study using analysis of repetitive DNA sequence by Piarroux R *et al.,* [1995] it was observed that, amongst the *leishmania* causing VL, *L. donovani* isolated from foci in which human beings are the main reservoirs clustered in an independent branch and by contrast, *L. infantum* and *L. chagasi* are canine parasites that rarely infect human beings and thus are clustered different.

A recent study by Mauricio I.L *et al.,* [1999] using three different approaches at different levels of resolution to explore the genetic information from *leishmania* species reveals a substantial amount of diversity within *L. donovani* complex. Further, RAPD

had grouped *L. donovani* strains according to the geographical origins, specifically Indian and Kenyan, showing a substantial divergence within taxon.

Genetic diversity is not only common in *L. donovani*, even in *L. major* which causes cutaneous leishmaniasis, strains isolated from the same geographical area show minor chromosomal size polymorphisms in their molecular karyotypes whereas strains from different geographical areas show more significant differences suggesting that the genomes of species of *leishmania* are quite plastic and that chromosomal rearrangements occurs during the evolution of various species [Samaras N *et al.*, 1987]. Currently a WHO sponsored genome mapping project on *L. major* is underway. Although it has been argued that the genome map of one strain would be applicable to another, there is very little evidence to substantiate this claim. Indeed, it is known that differences in gene copy number and organization differ between *L. donovani*, *L. chagasi*, *L. major* and other species. Moreover, it is difficult to reconcile the great differences in clinical symptoms caused by different species with identical genotype [Ghosh S.S., *et al.*, 1998]. For these reasons, it is necessary to characterize important genes, which has potential to be a diagnostic or vaccine or therapeutic candidate from different geographical regions. The assignment of the parasite species based alone on geographic location or the site of infection is not satisfactory. Accordingly, correct diagnosis and classification of pathogenic *Leishmania* isolate is essential to determine the clinical prognosis and a species-specific therapeutic approach [Marfurt J., *et al.*, 2003]. One such potential gene studied widely across different species from different geographical region is Gp63 a glycolipid-anchored zinc protease of 63kDa size [Webb J.R., *et al.*, 1991; Steinkraus HB *et al.*, 1993; Roberts S C *et al.*, 1993].

In India, VL is a serious problem in Bihar, west Bengal and eastern Uttar Pradesh where, there is under-reporting of Kala-azar (KA) and post kala-azar dermal leishmaniasis in women and children of 0-9 years of age. The recent epidemics in 1992 of VL killed more than 100,000 people in India and Sudan. Spraying of DDT helped control KA in India, however there are reports of the vector *Phlebotomus argentipes* developing resistance. Also, lymphadenopathy, a major presenting feature in India raises the possibility of a new vector or a variant of the disease [Bora D., 1999].

The Post kala-azar dermal leishmaniasis (PKDL) is a sequel to KA in India and Sudan; the disease develops months to years after the patient recovery from KA. Cutaneous lesions characterize the disease and they demonstrate great variability, ranging from

hypo-pigmented macules to erythematous papules and from nodules to plaques. As in leprosy, the wide clinical spectrum of PKDL reflects the immune response of the individual to the *leishmania* organism. Lesions may be numerous and persist for decades. Isolated parasites from the lesions are identical to those causing the original visceral disease.

The clinical and epidemiological findings in leishmaniases are no' pathognomic and these can mimic with several endemic conditions such as malaria, tuberculosis, syphilis and fungal infections. Hence, a laboratory diagnosis is required to confirm the clinical suspicion. The diagnostic tools used for each leishmanial syndrome viz. visceral, cutaneous, and mucocutaneous form, vary but the gold standard in each case remains the demonstration and isolation of the parasite from appropriate tissue [Singh S *et al.*, 2003].

The clinical signs and symptoms are not enough to differentiate VL from other similar conditions such as malaria, tropical splenomegaly syndrome schistosomiasis or cirrhosis with portal hypertension, African trypanosomiasis, milliary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma, and leukemia. Hence other diagnostic methods are required [Herwaldt BL, 1999; Davidson RN, 1998]. Amongst these the most specific and standard technique is parasitological demonstration or isolation of the causative agent. Marrow obtained from sternal or iliac crest puncture is a much safer but a painful method. The aspirates are smeared on the glass slide and stained with Romanowsky's stain to demonstrate the amastigote forms of the parasite. However; on culture it can give positive results in up to 80% of the cases. Lymph gland puncture gives positive results in 60% of the cases. Juice is extracted from any enlarged lymph gland and subjected to both direct examination and culture to give the best chance of diagnosis [Williams, J. E, 1995; Manson-Bahr PEC, 1987]. Primary isolation of *L. donovani* is made on solid Novy-MacNeal- Nicolle (NNN) medium having 20-30% rabbit blood or liquid Schneider's insect medium supplemented with 10% v/v foetal calf serum (FCS). Other suitable growth media can also be used particularly for maintaining the subcultures of the promastigotes using FCS or other supplements including human urine [Singh S *et al.*, 2000]. Demonstration of the parasites in the spleen and liver is one of the most accurate methods available to determine leishmanial infections. Ninety percent of the active cases show parasites in splenic and liver aspirates. The smallest needle possible,

preferably, 21-gauge (0.8 mm) should be used to minimize the risk of complications such as hemorrhage of the spleen [Williams, J. E, 1995]. Part of the splenic aspirate can be used to make smears for direct microscopic examination and the rest should be cultured. Liver biopsy material is less likely to demonstrate parasites on direct examination or on culture; however histological examination will show amastigotes in Kupffer cells in the portal system.

Occasional reports of finding the *Leishmania* parasites in blood in patients of Kala-azar from Kenya and India have been published. Blood in anticoagulant is centrifuged at 2000g for 10 min and the cells from the buffy coat removed and used to prepare smears and inoculate cultures. The amastigotes can be found in and around Macrophage cells. The volume used in culture inoculation is important, 1-3 drops on NNN or Schneider's medium has given successful results [Manson-Bahr PEC, 1987].

The conventional microscopic methods are invasive and painful carrying risk of iatrogenic infections and fatal hemorrhages. Though demonstration of the amastigote form of parasite in the tissues is being used since its discovery as a parasitic disease in 1903, it is least sensitive and unable to detect occult and sub clinical infections. The sub clinical and latent form of infection has become a major concern in recent years, as these can flare up due to immune suppression such as in HIV infection and the infection can be transmitted through organ transplants. Serological diagnosis is based on the presence of specific humoral response as in cases of visceral leishmaniasis or cell mediated immune response in cases of cutaneous and mucocutaneous leishmaniasis, evoked by the immune system against the causative pathogen. There are ranges of serological methods available for the diagnosis of VL varying in accuracy and specificity. These included non-specific and specific tests. With on-going research newer better methods are continually becoming available.

The formol gel test is oldest serological test and has the advantage of being cheap and simple to perform. Serum obtained from about 5 ml of blood is mixed with one drop of 30% formaldehyde. A positive reaction is shown if the mixture solidifies and forms a white opaque precipitate within 20 minutes. A positive test cannot be detected until 3 months after infection and becomes negative 6 months after cure. The test is non-specific since it is based on detecting raised levels of IgG and IgM immunoglobulins which are also raised in other infections such as African trypanosomiasis, malaria and

schistosomiasis etc. [WHO expert committee report, 1991]. Several other tests based on this principle had been in use in past but very rarely used these days. [Singh S, 1999]

There are number of specific serological tests and all have variable sensitivity and specificity for disease diagnosis. Some of these tests include indirect haemagglutination (IHA), counter current immunoelectrophoresis (CCIEP), Immunodiffusion (ID) etc. but all these tests are cumbersome and lack sensitivity and specificity and hence not commonly used. Some more commonly used ones are described below.

Leishmanin Skin Test (LST): Delayed hypersensitivity is an important feature of cutaneous forms of human leishmaniasis and can be measured by the leishmanin test, also known as the Montenegro reaction. Leishmanin is a killed suspension of whole (0.5-1 x 10^7/ml) or disrupted (250 μg protein/ml) promastigotes in pyrogen-free phenol saline. No cross-reactions occur with Chagas' disease, but some cross-reactions are found with cases of glandular tuberculosis and lepromatous leprosy. Leishmanin Skin Test is usually used as an indicator of the prevalence of cutaneous and mucocutaneous Leishmaniasis in human and animal populations and successful cure of the visceral leishmaniasis [Singh S, 1999, Sassi A, *et al.,* 1999]. During active kala-azar disease there will be no or negligible cell mediated immune response. However, the leishmanin antigen is not commercially available and no field study has been carried out in India.

2. Indirect fluorescent antibody test (IFAT): The Indirect fluorescent antibody test is one of the most sensitive tests available. The test is based on detecting antibodies, which are demonstrated in the very early stages of infection and undetectable six to nine months after cure. If the antibodies persist in low titers it is good indication of a probable relapse. Titers above 1/20 are significant and above 1/128 are diagnostic [Williams, J. E, 1995]. There is a possibility of a cross reaction with trypanosomal sera, however, this can be overcome by using *leishmania* amastigotes as the antigen instead of the promastigotes [Gari-Toussaint M, *et al.,* 1994].

3. Agglutination test: The DAT is a highly specific and sensitive test. It is cheap and simple to perform making it ideal for both field and laboratory use. The antigen is prepared from promastigotes *of L. donovani* and test can be carried out on plasma, serum, blood spots and whole blood. For long time DAT remained first line diagnostic tool in resource poor countries. The method uses whole, stained promastigotes either as a suspension or in a freeze-dried form. The freeze-dried form is heat stable and

facilitates the use of DAT in thc field. However, the major disadvantage of DAT is the relative long incubation time of 18 h and the need for serial dilutions of blood or serum [Schallig HD *et al.*, 2001]. Another major disadvantage of DAT is that it has no prognostic value for evaluating the parasitological cure of the disease, as the test may remain positive for several years after cure. Recently, Schoone *et al.*, [2001] have developed a fast agglutination-screening test for the rapid detection (<3h) of anti-leishmania antibodies in serum samples and on blood collected on filter paper. The FAST utilizes only one serum dilution leading to qualitative results. The FAST offers the advantages of the DAT based on the freeze-dried antigen with respect to stability of the antigen, reproducibility, specificity and sensitivity.

4. Immunoblotting: Serodiagnosis using immunoblotting has been attempted and reported superior and stage specific. The various antigens expressed during the course of infection can also be documented. It also has an added advantage of permanent documentation. However, the technique is not user friendly and limited only to research laboratories [Herwaldt BL., 1999; Singh S, 1999; Schallig HD *et al.*, 2001].

5. Antigen Detection: The detection of antigen in the patient's serum is complicated by the presence of high level of antibodies, circulating immune complexes, serum amyloid, rheumatoid factor and auto antibodies all of which may mask immunologically important antigenic determinants or competitively inhibit the binding of free antigen. Antigen detection test would, in principle provide better means of diagnosis of leishmaniasis. Since antigen levels are expected to broadly correlate with the parasite lo ad, the antigen detection may be an ideal alternative to the antibody detection in immunocompromised patients, where antibody response is very poor. Though a few reports are published, no satisfactory antigen detection system is currently available [Senaldi G *et al.*, 2001; Attar ZJ *et al.*, 2001]. Recently, a latex agglutination test (KATEX) for the detection of leishmanial antigens in the urine of patients with VL is developed. The results obtained with KATEX using samples collected from different foci of VL indicate that, the test works well regardless of the geographical origin of samples. The test had 100% specificity and sensitivity between 68-100% [Attar ZJ *et al.*, 2001]. Whether the test has applications for the detection of asymptomatic cases of VL and monitoring therapy is yet to be confirmed.

6. **Enzyme linked immunosorbent assay (ELISA):** The Enzyme Linked Immunosorbant Assay (ELISA) is a valuable tool in the serodiagnosis of leishmaniasis. The test is useful for laboratory analysis and field applications. The ELISA can be performed easily and is adaptable for use with purified or defined antigen. Immunodiagnosis is greatly influenced by the antigen used. The antigens used in the design of immunodiagnostic tests for leishmaniasis have traditionally been derived from promastigotes that have been cultivated in vitro or from recombinant proteins, alteration of the antigen used for ELISA and DAT from the whole promastigote or soluble antigens to more specific and potential recombinant leishmanial and peptide antigens have improved VL diagnosis [Senaldi G *et al.*, 2001]. Several antigen molecules have recently been reported [Martin SK *et al.*, 1998, Rajasekariah GH *et al.*, 2001]. The excretory, secretary and metabolic antigens (Ld-ESM), released by *L. donovani* promastigotes into a protein-free medium were used for the serodiagnosis of VL by ELISA. The Ld-ESM has been found to be 100% specific and sensitive, the Positive Predictive Value was 99.99% and Negative Predictive Value was 95.45%. However further retrospective and prospective multisite evaluation is required to validate these findings [Schoone GJ *et al.*, 2001]. Lately, a variety of recombinant antigens have been developed, recently a gene related to the *L. major* gene B encoding a hydrophilic protein expressed on the surface of both promastigotes and amastigotes of *L. major* characterized by an amino acid repeating motif of 5.5 copies of a 14-amino acid sequence has been identified and shown to be expressed in *L. donovani*. The protein encoded by *L. donovani* gene B homologue contains up to 22 copies of a repetitive element in which 9 out of 14 residues are completely conserved between the two species. The ELISA performed using repetitive peptide sequence of GBP from *L. donovani* and recombinant GBP of *L. donovani* as solid-phase ligand was developed. However the limitations of this antigen are that it can be used for serodiagnosis of visceral leishmaniasis only in areas endemic for *L. donovani* but not for areas that are co-endemic for other *Leishmania* species and the specificity and sensitivity are not very high [Jensen AT etal 1999].

Raj *et al.*, have developed another recombinant protein rORFF of *L. infantum* origin for diagnosis of VL in India. The ORFF protein is encoded in the LD1 locus of chromosome 35 of *L. infantum*, an ELISA with this antigen proved to be sensitive with as little as 5ng of rORFF when performed with different groups of patients like

confirmed VL, suspected VL, Intermittently treated endemic normal and non-endemic normal. Further the same groups were subjected to DAT using whole promastigote and ELISA using total soluble antigens, the ELISA using rORFF was found to be more sensitive than others. Although this antigen is highly sensitive and specific for VL, it also was found to be positive in 40% cases of confirmed CL due to *L. major* or *L. tropica*. Further the test is in early stage and needs to be evaluated by others and its utility for the field diagnosis is yet to be studied [Raj VS *et al.*, 1999]. In a recent study conducted in Mediterranean VL where *L. infantum* is the causative agent ten recombinant and purified antigens were evaluated using ELISA by Maalej *et al.*, [2003]. Of these the recombinant antigens rgp63, a major surface antigen of leishmania which is not present on *Trypanosoma cruzi* or other kinetoplasts and rGBP had good performance but not very sensitive and specific to be used for reliable diagnosis (<90%). It is suggested that the use of recombinant proteins from *L. infantum* rather than *L. major* as they done in that study would have yielded a better result.

Another recombinant antigen, belonging to the kinesin family of motor proteins, recombinant K39 (rK39) has been shown to be specific for antibodies arising during VL caused by members of the *L. donovani* complex, which include *Leishmania chagasi* and *L. infantum*. This antigen, which is a member of the kinesin family, encodes a protein with a repetitive epitope, consisting of 39 amino acid residues (K39) is highly sensitive and predictive for onset of acute disease and high antibody titers have been demonstrated in VL patients. In contrast, it shows no detectable anti-rk39 antibodies in cutaneous or mucocutaneous leishmaniasis. The antibody titers to this antigen directly correlate with active disease and have a tremendous potential as a means of monitoring chemotherapy and in predicting clinical relapse [Burns JM Jr *et al.*, 1993; Singh S *et al.*, 1995; Badaro R *et al.*, 1996; Singh S *et al.*, 2002; Maalej IA *et al.*, 2003, US PTO 5,411,865; US PTO 5,719,263]. In addition rk39 ELISA, has a high predictive value for detecting VL in immunocompromised persons, like AIDS patients [Houghton RL *et al.*, 1998]. This antigen is now commercially available in the form of antigen-impregnated nitrocellulose paper strips adapted for use under field conditions. The rK39 strip test has been found useful for the field diagnosis of Kala-azar in India [Sundar S *et al.*, 1998]. However; the same had markedly less sensitivity in Sudan [Zijlstra EE *et al.*, 2001].

Molecular Methods

Molecular biology is increasingly relevant to the diagnosis and control of infectious diseases. Information on DNA sequences has been extensively exploited for the development of polymerase chain reaction-based assays for the diagnosis of leishmaniasis and the identification of parasite species. Techniques such as micro arrays and nucleic acid sequence-based amplification will eventually allow rapid screening for specific parasite genotypes and assist in diagnostic and epidemiological studies.

The early diagnosis of leishmaniasis is important in order to avoid severe damage or death of the patient. The routine diagnosis of leishmaniasis relies on either the microscopical demonstration of *Leishmania* amastigotes in aspirates from lymphoid tissue, Liver or Bone marrow aspirates, in slit skin smears or peripheral blood or culturing. However, the retrieval of the sample is uncomfortable to the patient and the isolation of parasite by culturing is time consuming, difficult and expensive. The immunological methods fail to distinguish between past and present infections and are not reliable in the case of immunocompromised patients. Further more; none of the serological methods addresses the problem of species identification, which is important for determining appropriate diseases control measures. Patients with cutaneous (CL) or mucocutaneous leishmaniasis (MCL) often have low or no leishmania antibodies, because of the localized character of the disease, and thus serological tests are mostly negative. Molecular approach capable of detecting nucleic acids unique to the parasite in the tissue would address these limitations. Therefore, PCR is an important tool for the diagnosis of CL and MCL. PCR has also been reported very useful for the diagnosis of PKDL. A variety of DNA based detection methods targeting DNA and RNA genes have been developed. PCR has caused a revolution in the diagnosis of Leishmaniasis [Singh S *et al.*, 2003].

Amongst the molecular methods used for clinical diagnosis, PCR has proved to be highly sensitive and specific technique. A recent study has reported a PCR assay that could detect parasitemia a few weeks before the appearance of any clinical signs or symptoms. Different DNA sequences in the genome of leishmania like ITS region, gp63 locus, telomeric sequences, sequence targets in rRNA genes such as 18s rRNA

and SSU-rRNA and both conserved and variable regions in kDNA minicircles are being used by various workers [El Tai NO *etal.*, 2001; Pizzuto M *etal.*,2001; Wortman G *etal.*, Monroy Ostria & Sanchez-Tezeda G, 2002, Chiurillo MA *etal.*, 2001].

In a recent study comparing three different techniques such as PCR fingerprinting, PCR-RFLP and PCR SSCP to reveal the intraspecific polymorphism, the PCR-SSCP technique has been found to be advantageous than the other two for the detection of sequence variation in rRNA genes within the *L. donovani* species. In addition, it can be performed easily and rapidly without prior cultivation of the parasite facilitating detection and identification of the parasite simultaneously [El Tai NO *etal.*, 2001]. Another PCR assay assessed by Pizzuto *et al.*, for post therapeutic follow up and the detection of relapses was found 97% sensitive with peripheral blood and 100% sensitive with bone marrow for detecting leishmania species among HIV-infected patients using SSU rRNA gene target [Pizzuto M *etal.*,2001].

The recent development in PCR technology such as fluorogenic probes and automation takes care of non-specific amplification of sequences. In this technique, two fluorogenic dyes, a reporter dye and a quencher dye attach to 5' and 3' ends of the probes. At the end of PCR the fluorescence is directly proportional to the number of amplicons. The use of fluorogenic real-time PCR using SSU-rRNA gene added with complete automation has made it possible to quantitate the parasite burden on the host, besides being rapid, sensitive and highly specific [Wortman G *et al.*, 2001; Monroy Ostria & Sanchez-Tezeda G, 2002]. A novel PCR assay targeted at *L. donovani* telomeric sequences is specific and sensitive for *L. donovani* and *L. infantum*. It has increased specificity compared with kDNA-based assay, little cross-reactivity with *L. major* or *L. tropica* strains. Further, this PCR assay, in combination with the simple protocol for recovery of Giemsa-stained slides of bone marrow, proved useful for the retrospective study of archive material [Chiurillo MA *etal.*, 2001].

Several strains might circulate in an endemic area at a given time hence, species and strain specific primers have been developed to find genetic heterogeneity. Recently primers developed by the applicant could differentiate the Indian strains causing VL and PKDL forms. An Alu-PCR-like amplification was performed from the cultured *L. donovani* isolates from VL and PKDL patients. The banding pattern of the PCR

amplicons could clearly group all the PKDL strains in one group while VL strains had intra-species heterogeneity.

The applicants did extensive search of the patent database with different key words to study the previous work done on the Alu PCR / PCR based diagnosis of leishmaniasis and PCR amplification of the kinesin gene to diagnose and differentiate the VL and PKDL causing strains. Discussed below are the few US patents on the subject concerned and the uniqueness of the applicant's invention.

The United States Patent no. 5,411,865 by Reed in May 2, 1995 teaches about the method of detecting anti-leishmania parasite antibodies. The compound disclosed are method for detecting anti-Leishmania parasite antibodies to a 230 kDa antigen present in Leishmania chagasi and Leishmania donovani which comprises obtaining a sample from an individual, contacting the sample with a recombinant K39 repeat unit antigen comprising the amino acid sequence as shown in SEQ ID NO: 3, and detecting the presence of anti-Leishmania parasite antibodies in the sample which bind to the recombinant K39 repeat unit antigen.

The United States Patent no. 5,719,263 by Reed in February 17, 1998 teaches about the 230Kd antigen present in Leishmania species. The compound disclosed is an isolated 230 Kd antigen that is present in Leishmania chagasi and Leishmania donovani, and isolated polypeptides comprising one or a plurality of K39 repeat antigens. Also disclosed are DNAs encoding the 230 Kd antigen and the K39 repeat antigen, and vaccine compositions comprising the antigens.

The above disclosed 230kDa antigen and the isolated polypeptide comprising the K39 repeats are only serological methods and further reported to be not very sensitive in certain geographical areas where VL is highly endemic and caused by *L. donovani*. In contrary, the applicant's invention provides methods and compounds, which deal with molecular diagnostic or Nucleic acid amplification based tests.

The United States Patent no.5, 912,166 by Reed, *et al.*, in June 15, 1999 teaches about compounds and methods for diagnosis of leishmaniasis infection. The compounds provided include polypeptides that contain at least an epitope of the Leishmania chagasi acidic ribosomal antigen LcP0, or a variant thereof. Such compounds are useful in a

variety of immunoassays for detecting Leishmania infection and for identifying individuals with asymptomatic infections that are likely to progress to acute visceral leishmaniasis. The polypeptide compounds are further useful in vaccines and pharmaceutical compositions for preventing leishmaniasis.

However, the applicant's present invention does not deal with acidic ribosomal antigen LcPO.

The United States Patent No. 6,638,517 by Reed, *et al.*, in October 28, 2003, Leishmania antigens for use in the therapy and diagnosis of leishmaniasis teaches compositions and methods for preventing, treating and detecting leishmaniasis and stimulating immune responses in patients. The compounds provided include polypeptides that contain an immunogenic portion of one or more Leishmania antigens, or a variant thereof. The patent also discloses vaccines and pharmaceutical compositions comprising such polypeptides, or polynucleotides encoding such polypeptides, are also provided and may be used, for example, for the prevention and therapy of leishmaniasis, as well as for the detection of Leishmania infection.

United States Patent Application 20030162182, Salotra Poonam *et al.*, August 28, 2003, Species-specific PCR assay for detection of Leishmania donovani in clinical samples of kala-azar and post kala-azar dermal leishmaniasis teaches methods and compounds for the polymerase chain reaction (PCR) assay for the diagnosis of leishmaniasis using specific oligonucleotide primers for the identification of *Leishmania donovani* parasites in clinical samples.

The applicant's invention uses a target in the genomic DNA of *leishmania* that is entirely different from the Salotra Poonam's *et al.*, work, where they amplify the minicircles in the kinetoplast DNA which is a type of mitochondrial DNA. Where as, the applicant's invention uses genomic DNA as a target for the PCR amplification.

The present invention provides a method of detecting Leishmaniasis by amplification of the conserved repeat region of the kinesin related antigen gene, whereas all the other reported methods are based on the polypeptide derived from the kinesin related antigen gene. Another feature of this invention is that, the PCR method can differentiate between visceral Leishmanisis (VL) and post kala-azar dermal leishmaniasis (PKDL).

OBJECTS OF THE INVENTION

The main object of the present invention is to develop a PCR amplification method to detect Leishmaniasis in the patients infected with *L. donovani* strains based on the conserved repeat region of kinesin gene of *Leishmania*.

Another object of this invention is to provide primers for PCR method to detect leishmaniasis in the patients infected with *L. donovani* strains based on the conserved repeat region of kinesin gene of *Leishmania*.

Another object of this invention is to provide a PCR amplification method to differentiate between VL and PKDL forms of the disease.

DESCRIPTION OF THE INVENTION

The protozoan parasites of the genus *Leishmania* are the causative agents of visceral leishmaniasis (VL), also called kala-azar (KA). KA is a symptomatic infection of the liver, spleen and bone marrow caused by organisms of *Leishmania donovani* complex. PKDL (Post kala-azar dermal leishmaniasis) is an unusual dermatosis that develops as a sequel of KA, producing gross cutaneous lesions in the form of hypopigmented macules, erythema and nodules. The disease is relatively common in the Indian subcontinent and less frequent in East Africa, but exceptional in the American and European continents. Detection and characterization of *Leishmania* from patients of both KA and PKDL is important for deciding treatment regimens as well as for understanding the disease epidemiology. In many patients the dermal manifestations are seen even when the patient never had visceral form hence the term post-kala-azar dermal leishmaniasis is a misnomer. It is also seen that no kala-azar patient has ever developed PKDL once he/she has migrated to a PKDL non-endemic area after kala-azar treatment. Therefore, the applicant proposed a hypothesis that VL and PKDL causing strains of *Leishmania donovani* are different. To elucidate the proposed hypothesis, the applicant successfully designed and standardized an Alu-PCR and its primers to differentiate between these two strains.

The present invention provides a unique PCR amplification technique to amplify the Kinesin gene of different Indian isolates of *L. donovani*. This has been developed by the applicant to analyze genetic difference of the strains causing VL and PKDL on the basis of number of bands in the ladder of PCR products, on agarose gel electrophoresis, between the strains of *Leishmania donovani* that cause visceral leishmaniasis and

strains that cause post kala-azar-dermal leishmaniasis, using the following sets of PCR primers:

Forward Primer (SS-KIN 1):
5' CTAGAGCAGCAGCTTCG 3' (17 oligomer)
Forward Primer (SS-KIN 3):
5' CTTGAGCAGCAGCTTCG 3' (17 oligomer)
Reverse Primer (SS-KIN 2):
5' CGTGGCCCTCGTGTTCT 3' (17 oligomer)
Reverse Primer (SS-KIN 4)
5' CGCGGCCCTCGTGTCCT 3' (17 oligomer)

The invention further provides a method to differentiate VL and PKDL strains using an Alu-PCR using the above primers sets named as SS-KIN1, SS-KIN2, SS-KIN3 and SS-KIN4, which are designed on the basis of consensus, repetitive 117bp sequences in the Kinesin related gene of *Leishmania donovani* strain MHOM/IN/DD8.

Parasites were initially isolated as Promastigotes in NNN medium from clinical samples of Kala-azar and post Kala-azar dermal leishmaniasis patients and subsequently adapted to grow at 25^0C in Medium 199 containing 10% heat inactivated FCS. For routine maintenance, samples of the inoculum containing parasites were introduced aseptically into culture tubes with 4ml of medium 199 supplemented with 10% FCS. The tubes were placed in cooled incubator at 25^0C and the growth was monitored at regular intervals by microscopy. For mass cultivation of the parasite, samples of inoculum containing parasites were introduced aseptically into 200ml of M199 containing 10%FCS in a 500ml tissue culture flask and incubated in a cooled incubator at 25^0C until mid log phase (7-10 days). The parasites were then harvested and used for nuclear DNA isolation.

The parasites in their mid log phase was harvested by centrifuging at 5000 rpm in a refrigerated centrifuge. Parasite nuclear DNA was isolated following standard protocol [Lu H.G. *et al.*, 1994] with minor modifications. Approximately 1-5 X 10^9 promastigotes were lysed in 10 volumes of lysis buffer (NaCl, 100 mM, Tris-HCl, 10mM (pH 8.0), EDTA 10mM, Proteinase K/ml 100μg, Sarcosyl 1.5%) at 60^0 C for 3 hours. The kinetoplast DNA networks were sedimented by centrifugation at 27,000 X g for 1 hour and resuspended in TE buffer (Tris-HCl (pH 8.0) 10mM, EDTA (pH 8.0) 1mM). The nuclear DNA was isolated from the supernatants left after sedimentation of the kinetoplast DNA. These supernatants were incubated overnight for further digestion

of proteins at 65^0 C. The nuclear DNA was subjected to several cycles of phenol/chloroform extractions by adding equal volume of phenol/chloroform mixture, mixing thoroughly followed by sedimentation by centrifugation at 5000 rpm for 15 minutes. The nuclear DNA was precipitated by adding $1/10^{th}$ the volume of 3M-sodium acetate and 2 volumes of 100% ethanol mixed well and incubated at -20^0C for 1 hour. The mixture was sedimented by centrifugation at 5000 rpm for 30 minutes at 4^0C. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer. The concentration and purity of the DNA was measured by taking OD at 260/280nm.The DNA was stored at -70^0C until use.

The VL and PKDL strains, as described herein were isolated from various parts of India (Table 1) and maintained in Medium 199 supplemented with 10% fetal calf serum and mass culture propagation for DNA isolation for PCR were done in medium 199 with 5% FCS +5% human urine (post menopausal female) and culture flasks incubated with agitation at $17\text{-}20^0$C in a BOD incubator,

Table1:

	Strain ID	Source	Geog. Location	Disease
1.	HM/IN/DD8	WHO std. strain	Bihar	VL
2.	HM/IN/UR6,	IICB, Calcutta	West Bengal	VL
3.	HM/IN/Ag83,	IICB, Calcutta	West Bengal	VL
4.	HM/IN/SS,	PGIMER, Chd.	Bihar	VL
5.	HM/IN/LD183,	Our Lab, AIIMS	Bihar	VL
6.	HM/IN/KE16,	Our Lab, AIIMS	Bihar	VL
7.	HM/IN/J1,	Our Lab, AIIMS	Bihar	VL
8.	HM/IN/J2,	Our Lab, AIIMS	Bihar	VL
9.	HM/IN/J3,	Our Lab, AIIMS	Bihar	VL
10.	HM/IN/RMRI,	RMRI, Patna	Bihar	PKDL
11.	HM/IN/RMP7,	RMRI, Patna	Bihar	PKDL
12.	HM/IN/RMP8,	RMRI, Patna	Bihar	PKDL
13.	HM/IN/RMP142,	RMRI, Patna	Bihar	PKDL
14.	HM/IN/RMP155,	RMRI, Patna	Bihar	PKDL
15.	HM/IN/RMP240,	RMRI, Patna	Bihar	PKDL
16.	HM/IN/RS,	IICB, Calcutta	Not known	VL
17.	HM/IN/MF,	IICB, Calcutta	Not known	VL
18.	HM/IN/GEI,	IICB, Calcutta	Not known	VL
19.	HM/IN/GEIV	IICB, Calcutta	Not known	VL

This PCR has been standardized using the DNA isolated from the culture maintained VL and PKDL isolates of *Leishmania donovani* and then adapted to amplify with the whole blood sample of leishmaniasis patients.

The Alu-PCR was carried out using the indigenously designed primers SS-KIN1, SS-KIN2, SS-KIN3 and SS-KIN 4, which are developed based on the consensus, repetitive 117bp sequences in the Kinesin related gene of *Leishmania donovani*. SS-Kin PCR strategy was based on the data concerning structure and organization of repetitive elements in the human genome and similarly in the *Leishmania* and hence is called Alu-PCR [Piarroux R *et al.*, 1993]. It has been observed that under certain conditions, probes originating from repetitive sequences recognize a locus in a specific manner likewise the primers SS-KIN1, 2, 3 & SS-KIN 4 bind in strain specific manner.

The Alu- PCR using the primers sets named as SS-KIN1, SS-KIN2, SS-KIN3 and SS-KIN4, which are designed on the basis of consensus, repetitive 117bp sequences in the Kinesin related gene of *Leishmania donovani* strain MHOM/IN/DD8 was carried out as follows:

Approximately 100ng of nuclear DNA from different isolates were amplified in a multiplex PCR for 36 cycles in 50 μl reaction mixtures, each containing, 200 μM each of deoxynucleoside triphosphates (dNTPs), 1.5 units of *Taq DNA Polymerase* (Perkin Elmer) and 5 μl of 10 X PCR buffer (100 mM TAPS (pH 8.8), 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin). The working concentration of each primer was 0.5 μM. The temperature cycles used were: 94 ^{0}C for 10 min; 94 ^{0}C for 60s, 52 ^{0}C for 60s, 72 ^{0}C for 60s followed by 72 ^{0}C for 10 min. The PCR products were electrophoresed in 2.5% agarose gel, stained with Ethidium Bromide and visualised on an UV-transilluminator to identify the banding pattern difference amongst the strains of VL and PKDL obtained from clinical samples.

All the four primers were used for amplification at equimolar concentration in a single Alu-PCR reaction mixture. It has been observed that under certain conditions, probes originating from repetitive sequences recognize a locus in a specific manner; likewise the primers SS-KIN1, 2, 3 & SS-KIN 4 bind in strain specific manner. The ladder of amplified products showed distinct pattern of bands for VL strains and PKDL strains, in which the number of unique bands for VL strains were 8-10 and for PKDL strains 12-14, and most of the PCR amplicons were in the size range of 0.4-1.5 kb from VL strains while in the MW size range of 0.22- 1.5 kb from PKDL strains as detailed in the description.

After disclosing the primers to differentiate the two causative strains of Leishmania, the applicant found that dermal manifestations of Leishmaniasis in Bihar and adjoining areas are due to in-vivo hybridization and development of quasi species.

PCR amplification of the kinesin gene of different Indian isolates of *L. donovani* were carried out following the method exclusively developed by the PI using the self designed primers SS-KIN-1, 2,3 and SS-KIN-4. These primers were designed in such a way that, an 117bp fragment of the kinesin gene is amplified and a ladder is always

seen depending on the number of repeats and copy number of the kinesin gene. The PCR products on electrophoresis in 2.0% agarose gel, stained with Ethidium Bromide on visualisation under an UV-transilluminator (UVP), are observed as ladders of amplicons.

The amplicon banding patterns were found different for VL isolates and PKDL isolates. The number of bands in the ladder of amplicons varied from 5-10 for visceralising isolates while in PKDL isolates it was between 12-14 (figure 1).

These results, suggests that VL and PKDL causing agents are genetically different. The following factors need consideration viz.

1.) PKDL being considered to be the sequel of infection with *Leishmania donovani*

2.) The presence of kinesin gene conserved only in visceralising species but minor genotypic differences between VL and PKDL isolates imply that, PKDL may be due to recombination between the two *Leishmanial species* co-infecting the same host and then evolving a new strain causing PKDL.

Alternatively, it can be hypothesised that in some cases the *L. donovani* that caused Kala-azar might acquire more copies of kinesin gene as is evidenced from the more bands of amplicons and this genotype transformation makes these mutated strain more dermotrophic.

Widespread use of multiplex PCR using SS-KIN 1,2,3 & 4 primers is to diagnose *Leishmania donovani* infection and to differentiate the strains whether these will cause visceral form of Leishmaniasis (kala-azar) or a dermal form of Leishmaniasis in Bihar commonly known as PKDL.

The DNA from clinical samples is extracted by adding 25µl of patient whole blood to 1 ml of sterile distilled water in 1.5ml Microfuge tube followed by mixing and incubation at room temperature for 30 minutes, centrifuged at 12,000 rpm for 5 minutes, carefully removed all but 30-50 µl of supernatant and added 200 µl of Instagene Matrix (Bio-Rad, USA) to the tube, after incubation at 56^0C for 30 minutes, the contents vortexed at high speed for 10 seconds, heated at 100^0 C in a heating block for 5 minutes, vortexed again and finally re-centrifuged at 12,000 rpm for 5 minutes, 20µl of the isolated DNA

from the supernatant was taken for PCR. Further, in the experiment 100-150 ng of nuclear DNA from different isolates were amplified for 36 cycles in 50 μl reaction mixtures, each containing, 200 μM each of deoxynucleoside triphosphates (dNTPs), 1.5 units of *Taq DNA Polymerase* (Perkin Elmer) and 5 μl of 10 X PCR buffer (100 mM TAPS (pH 8.8), 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin). The working concentration of each primer was 0.5 μM. The temperature cycles used were: 94 ^{0}C for 10 min; 94 ^{0}C for 60s, 52 ^{0}C for 60s, 72 ^{0}C for 60s followed by 72 ^{0}C for 10 min. The PCR products were electrophoresed in 1.5%- 2.5% agarose gel, stained with Ethidium Bromide and visualised on an UV-transilluminator.

The ladder of amplified products showed distinct pattern of bands for VL strains and PKDL strains, in which the number of unique bands for VL strains were 8-10 and for PKDL strains 12-14, and the PCR product of using the above mentioned primer sets is depicted here (figure 1). The figure clearly shows difference in banding pattern between the PKDL and VL strains. One can easily note that Leishmania strains from kala-azar (visceral leishmaniasis) patients show very few bands numbering 8-10 of 0.4-1.5 kb size. One can also make out easily that out of these many bands only two bands of ~ 0.8 and 0.85 kb sizes are most prominent.

In case of strains from PKDL (dermal leishmaniasis of Bihar) the number of PCR bands was significantly more. The number was between 12 and 14. All these PCR amplicons bands were seen between the sizes of 0.22-1.5kb. The most prominent were 4 bands of sizes ~6.0, 7.0. 8.0 and 1.5 kb.

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Dated this 22nd day of December, 2003

V.Lakshmikumaran

Attorney for the Applicants

To

The Controller of Patents

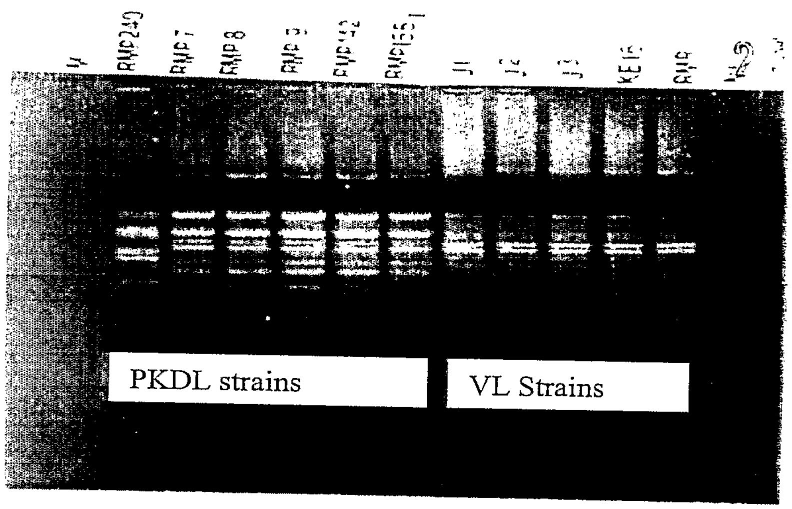
Patent Office

Delhi

1598-DEL-03

23 DEC 2003

Figure 1



FORM 2

22 DEC 2004

THE PATENTS ACT, 1970

(39 of 1970)

COMPLETE SPECIFICATION

(See section 10)

DUPLICATE

"Oligonucleotides for Detection of Leishmaniasis and methods thereof"

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, Divisional of Clinical Microbiology, Dept of Laboratory Medicine, Ansari Nagar, New Delhi -110 029, India and

DEPARTMENT OF BIOTECHNOLOGY, a Dept. of Govt. of India, CGO Complex, Lodhi Road, New Delhi 110 003

The following specification particularly describes the nature of this invention and the manner in which it is to be performed

"OLIGONUCLEOTIDES FOR DETECTION OF LEISHMANIASIS AND METHODS THEREOF"

TECHNICAL FIELD

The present invention provides a method to detect Leishmaniasis by amplification of the tandem repeat region of Kinesin-related gene from various strains of *L. donavani.* This invention also provides novel primers and an amplification method using these primers to detect and differentiate visceral leishmaniasis (VL) and post kala-azar-dermal leishmaniasis (PKDL) causing strains of *L. donavani.*

BACKGROUND AND PRIOR ART REFERENCES

Leishmaniasis, a vector-borne parasitic disease, is caused by obligate intramacrophage protozoa. It is characterized by diversity and complexity. It presents itself with a wide range of clinical forms. However, there are mainly 4 clinical forms. The Visceral Leishmaniasis (VL), also known as *kala azar*, is the most severe form of the disease, which, if untreated, has a mortality rate of almost 100%. The Cutaneous Leishmaniasis (CL) produces skin ulcers on the exposed parts of the body, such as the face, arms and legs. The number of ulcers may vary from 1 to as many as 200 in some cases, causing serious disability and leaving the patient permanently scarred. The third form is Mucocutaneous Leishmaniasis (MCL), or *espundia*. It can lead to extensive and disfiguring destruction of mucous membranes of the nose, mouth and throat cavities and can involve even the cartilage. The cutaneous form may lead to disseminated form, known as Diffuse Cutaneous Leishmaniasis (DCL). Leishmaniasis is caused by a total of about 21 species, which are transmitted by about 30 species of *Phlebotomine* sand flies [Herwaldt BL., 1999].

Leishmaniasis is presently endemic in 88 countries on five continents: Africa, Asia, Europe, North America and South America and a total of 350 million people are at risk of infection. It is estimated that worldwide 12 million people are affected by leishmaniasis; this figure includes cases with overt disease and those with no apparent symptoms. Of the 1.5-2 million new cases estimated to occur annually, only 600 000 are officially declared. Of the 500 000 new cases of VL, which occur annually, 90%, are in five developing countries: Bangladesh, Brazil, India, Nepal and Sudan. About 90% of all cases of MCL occur in Bolivia, Brazil and Peru and 90% of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1-1.5 million new cases reported annually

worldwide. The geographical distribution of leishmaniasis is limited by the distribution of the sand fly, its susceptibility to cold climates, its tendency to take blood from humans or animals and its capacity to support the internal development of specific species of *Leishmania* [Desjeux P 2001].

In India, VL is a serious problem in Bihar, West Bengal and Eastern Uttar Pradesh where, there is under-reporting of Kala-azar (KA) and post kala-azar dermal leishmaniasis in women and children between 0-9 years of age. The recent epidemics in 1992 of VL killed more than 100,000 people in India and Sudan. Spraying of DDT helped control KA in India, however there are reports of the vector *Phlebotomus argentipes* developing resistance. Also, lymphadenopathy, a major presenting feature in India raises the possibility of a new vector or a variant of the disease [Bora D., 1999].

The post kala-azar dermal leishmaniasis (PKDL) is a sequel to KA in India and Sudan; the disease develops months to years after the patient recovery from KA. Cutaneous lesions characterize the disease and they demonstrate great variability, ranging from hypo-pigmented macules to erythematous papules and from nodules to plaques. As in leprosy, the wide clinical spectrum of PKDL reflects the immune response of the individual to the *leishmania* organism. Lesions may be numerous and persist for decades. Isolated parasites from the lesions are identical to those causing the original visceral disease.

The clinical and epidemiological findings in leishmaniasis are not pathgnomic and these can mimic several endemic conditions such as malaria, tuberculosis, syphilis and fungal infections. Hence, a laboratory diagnosis is required to confirm the clinical suspicion. The diagnostic tools used for each leishmanial syndrome viz. visceral, cutaneous, and mucocutaneous form vary, but the gold standard in each case remains the demonstration and isolation of the parasite from appropriate tissue [Singh S *et al.*, 2003].

The clinical signs and symptoms are not enough to differentiate VL from other similar conditions such as malaria, tropical splenomegaly syndrome schistosomiasis or cirrhosis with portal hypertension, African trypanosomiasis, milliary tuberculosis, brucellosis, typhoid fever, bacterial endocarditis, histoplasmosis, malnutrition, lymphoma, and leukemia. Hence other diagnostic methods are required [Herwaldt BL, 1999; Davidson RN, 1998]. Amongst these the most specific and standard technique is parasitological demonstration or isolation of the causative agent. Marrow obtained from sternal or iliac crest puncture is a much safer but a painful method. The aspirates are smeared on the glass slide and stained with Romanowsky's stain to demonstrate the amastigote forms of the

parasite. However; on culture it can give positive results in up to 80% of the cases. Lymph gland puncture gives positive results in 60% of the cases. Juice is extracted from any enlarged lymph gland and subjected to both direct examination and culture to give the best chance of diagnosis [Williams, J. E, 1995; Manson-Bahr PEC, 1987]. Primary isolation of *L. donovani* is made on solid Novy- MacNeal- Nicolle (NNN) medium having 20-30% rabbit blood or liquid Schneider's insect medium supplemented with 10% v/v foetal calf serum (FCS). Other suitable growth media can also be used particularly for maintaining the subcultures of the promastigotes using FCS or other supplements including human urine [Singh S *et al.*, 2000]. Demonstration of the parasites in the spleen and liver is one of the most accurate methods available to determine leishmanial infections. Ninety percent of the active cases show parasites in splenic and liver aspirates [Williams, J. E, 1995]. Part of the splenic aspirate can be used to make smears for direct microscopic examination and the rest should be cultured. There are several methods for the detection of this disease in patients. The conventional microscopic methods are invasive and painful carrying risk of iatrogenic infections and fatal hemorrhages.

The formol gel test is oldest serological test and has the advantage of being cheap and simple to perform. This test is non-specific since it is based on detecting raised levels of IgG and IgM immunoglobulins [WHO expert committee report, 1991]. Several other tests based on this principle had been in use in past but very rarely used these days. [Singh S, 1999]

There are number of specific serological tests and all have variable sensitivity and specificity for disease diagnosis. Some of these tests include indirect haemagglutination (IHA), counter current immuno electrophoresis (CCIEP), Immuno diffusion (ID) etc. but all these tests are cumbersome and lack sensitivity and specificity and hence not commonly used. Some more commonly used ones are given below:

1. Leishmanin Skin Test (LST) [Singh S, 1999, Sassi A, *et al.*, 1999]
2. Indirect fluorescent antibody test (IFAT) [Williams, J. E, 1995, Gari-Toussaint M, *et al.*, 1994]
3. Direct Agglutination test [Schallig HD *et al.*, 2001]
4. Immunoblotting [Herwaldt BL., 1999; Singh S, 1999; Schallig HD *et al.*, 2001]
5. Antigen Detection [Senaldi G *et al.*, 2001; Attar ZJ *et al.*, 2001].
6. Enzyme linked immunosorbent assay (ELISA) [Martin SK *et al.*, 1998, Rajasekariah GH *et al.*, 2001, Schoone GJ *et al.*, 2001]

Raj *et al.* (1999) have developed a recombinant protein r-ORFF of *L. infantum* origin for diagnosis of VL in India. The ORFF protein is encoded in the LD1 locus of chromosome 35 of *L. infantum*, an ELISA with this antigen proved to be sensitive with as little as 5ng of r-ORFF when performed with different groups of patients like confirmed VL, suspected VL, Intermittently treated endemic normal and non-endemic normal. Further the test is in early stage and needs to be evaluated by others and its utility for the field diagnosis is yet to be studied [Raj VS *et al.*, 1999].

Another recombinant antigen, belonging to the kinesin-related gene family of motor proteins, recombinant K39 (rK39) has been shown to be specific for antibodies arising during VL caused by members of the *L. donovani* complex, which include *Leishmania chagasi* and *L. infantum*. This antigen, which is a member of the kinesin-related gene family, encodes a protein with a repetitive epitope, consisting of 39 amino acid residues (K39) is highly sensitive and predictive for onset of acute disease and high antibody titers have been demonstrated in VL patients. [Burns JM Jr *et al.*, 1993; Singh S *et al.*, 1995; Badaro R *et al.*, 1996; Singh S *et al.*, 2002; Maalej IA *et al.*, 2003, US PATENT No 5,411,865; US PATENT No 5,719,263]

Molecular Methods

Molecular biology is increasingly relevant to the diagnosis and control of infectious diseases. Information on DNA sequences has been extensively exploited for the development of Polymerase chain reaction-based assays for the diagnosis of leishmaniasis and the identification of parasite species. Techniques such as micro arrays and nucleic acid sequence-based amplification will eventually allow rapid screening for specific parasite genotypes and assist in diagnostic and epidemiological studies.

The early diagnosis of leishmaniasis is important in order to avoid severe damage or death of the patient. The routine diagnosis of leishmaniasis relies on either the microscopical demonstration of *Leishmania* amastigotes in aspirates from lymphoid tissue, Liver or Bone marrow aspirates, in slit skin smears or peripheral blood or culturing. However, the retrieval of the sample is uncomfortable to the patient and the isolation of parasite by culturing is time consuming, difficult and expensive. The immunological methods fail to distinguish between past and present infections and are not reliable in the case of immunocompromised patients. Furthermore, none of the serological methods addresses the problem of species identification, which is important for determining

appropriate diseases control measures. Patients with cutaneous (CL) or mucocutaneous leishmaniasis (MCL) often have low or no leishmania antibodies, because of the localized character of the disease, and thus serological tests are mostly negative. Molecular approach capable of detecting nucleic acids unique to the parasite in the tissue would address these limitations. Therefore, PCR is an important tool for the diagnosis of CL and MCL. PCR has also been reported very useful for the diagnosis of PKDL. A variety of DNA based detection methods targeting DNA and RNA genes have been developed. PCR has caused a revolution in the diagnosis of Leishmaniasis [Singh S *et al.*, 2003].

Amongst the molecular methods used for clinical diagnosis, PCR has proved to be a highly sensitive and specific technique. A recent study has reported a PCR assay that could detect parasitemia a few weeks before the appearance of any clinical signs or symptoms. Different DNA sequences in the genome of leishmania like ITS region, gp63 locus, telomeric sequences, sequence targets in rRNA genes such as 18s rRNA and SSU-rRNA and both conserved and variable regions in kDNA minicircles are being used by various workers [El Tai NO *etal.*, 2001; Pizzuto M etal., 2001; Wortman G *etal.* 2001, Monroy Ostria & Sanchez-Tezeda G, 2002, Chiurillo MA *etal.*, 2001]. Using PCR methodology, it is no more essential to undergo invasive methods such as bone marrow, splenic punctures, lymphnode biopsy, liver biopsy etc. or collect large volumes of blood samples. Even a few drops of blood on filter paper may be sufficient. [Da Silva *et al.*, 2004]

In a recent study comparing three different techniques such as PCR fingerprinting, PCR-RFLP and PCR SSCP to reveal the intraspecific polymorphism, the PCR-SSCP technique has been found to be advantageous than the other two for the detection of sequence variation in rRNA genes within the *L. donovani* species. In addition, it can be performed easily and rapidly without prior cultivation of the parasite facilitating detection and identification of the parasite simultaneously [El Tai NO *et al*, 2001]. Another PCR assay assessed by Pizzuto *et al.*(2001), for post therapeutic follow up and the detection of relapses, was found 97% sensitive to peripheral blood and 100% sensitive to bone marrow for detecting leishmania species among HIV-infected patients using SSU rRNA gene target [Pizzuto M *etal.*,2001]. However there are 2 major disadvantages of SSCP. First, the amounts of mobility differences have little if any correlation to the amount of sequence differences. Thus, the only information that can be gained from SSCP is if PCR amplicons are "identical" or different. Second, the optimal amplicon size for detection of most point mutations is rather small, around 200 bp. The strategies to deal with this limitation (e.g.

dideoxy fingerprinting or cutting amplicons with restriction enzymes) are often tedious and do not necessarily give the results desired.

Multiplex PCR in diagnosis and species identification of leishmaniasis: PCR can offer a rapid, sensitive, specific, and low-cost alternative. A number of PCR assays for identification of *Leishmania* at the genus level or for characterization of individual complexes of *L. braziliensis*, *L. mexicana* or *L. donovani* have been described. However, none of these PCR protocols identifies all three complexes in one assay.

Recently in last few years development in the field of molecular biology has led to the development of a simple sensitive and specific one step PCR based assay for differentiating the three complexes of New World *Leishmania*. This method employs different set of primers in a single PCR reaction and known as multiplex PCR. There is a report of use of this method, using the multicopy spliced leader (SL) RNA (mini exon gene) as a target. This assay generates species-specific products of different sizes for *L. braziliensis*, *L. mexicana*, and *L. donovani* and is suitable for use in non-sophisticated laboratories in countries where leishmaniasis is endemic. In another study *Leishmania* strains were characterized using a single 5' primer and two 3' primers combined in a single multiplex reaction. [Harris *et.al.* 1998, Belli *et.al.* 1998]. Although this method is useful but the chances of non-specific amplification or false positive results are high because of use of multiple primer sets. So, in order to avoid such results, one must select the primers amplifying highly conserved region in the species to avoid non specific amplification.

Several strains might circulate in an endemic area at a given time. Hence, species and strain specific primers have been developed to detect genetic heterogeneity. Recently primers developed by our group could differentiate the Indian strains causing VL and PKDL forms. A multiplex Alu-PCR-like amplification was performed with the cultured *L. donovani* isolates from VL and PKDL patients. The banding pattern of the PCR amplicons could clearly group all the PKDL strains in one group while VL strains had intra-species heterogeneity.

The applicants did extensive search of the patent database with different key words to study the previous work done on the Alu PCR / PCR based diagnosis of leishmaniasis and PCR amplification of the kinesin-related gene to diagnose and differentiate the VL and PKDL causing strains. Discussed below are the few US patents on the subject concerned and the uniqueness of the applicant's invention.

The United States Patent no. 5,411,865 by Reed in May 2, 1995 teaches about the method of detecting anti-leishmania parasite antibodies. The compound disclosed is for a method for detecting anti-Leishmania parasite antibodies to a 230 kDa antigen present in Leishmania chagasi and Leishmania donovani.

The United States Patent no. 5,719,263 by Reed in February 17, 1998 teaches about the 230Kd antigen present in Leishmania species. The compound disclosed is an isolated 230 Kd antigen that is present in Leishmania chagasi and Leishmania donovani, and isolated polypeptides comprising one or a plurality of K39 repeat antigens. Also disclosed are DNAs encoding the 230 Kd antigen and the K39 repeat antigen, and vaccine compositions comprising the antigens.

The above disclosed 230kDa antigen and the isolated polypeptide comprising the K39 repeats are only serological methods and further reported to be not very sensitive in certain geographical areas where VL is highly endemic and caused by *L. donovani*. In contrary, the applicant's invention provides methods and compounds, which deal with molecular diagnostic or Nucleic acid amplification based tests.

The United States Patent no. 5,834,592 by Reed *et al.*, in November 10, 1998 gives information about a an isolated polypeptide comprising an immunogenic portion of a *Leishmania* antigen having the amino acid sequence recited in SEQ ID NO: 4, or a variant of said antigen that differs only in conservative substitutions, modifications or combinations thereof. The antigen is considered to be important in immunodiagnosis and therapy of leishmaniasis. However the applicant invention differs from the compound patented.

The United States Patent no. 5,846,748 by Mandal *et al.*, in December 8, 1998 gives information about method for diagnosing visceral leishmaniasis in a patient by identification of a new key marker namely 9-O-acetylated sialoglycoconjugate. This invention relates to identification of a new key marker namely 9-O-Acetylated sialoglycoconjugate with the help of a known 9-O acetylsialic acid binding lectin, Achatinin-H useful for the diagnosis of visceral leishmaniasis, by a rapid, accurate haemagglutination assay. In contrary, the applicant's invention provides methods and compounds, which deal with molecular diagnostic or Nucleic acid amplification based tests.

The United States Patent no.5, 912,166 by Reed, *et al.*, in June 15, 1999 teaches about compounds and methods for diagnosis of leishmaniasis infection. The compounds

provided include polypeptides that contain at least an epitope of the Leishmania chagasi acidic ribosomal antigen LcP0, or a variant thereof. Such compounds are useful in a variety of immunoassays for detecting Leishmania infection and for identifying individuals with asymptomatic infections that are likely to progress to acute visceral leishmaniasis. The polypeptide compounds are further useful in vaccines and pharmaceutical compositions for preventing leishmaniasis. However, the applicant's present invention does not deal with acidic ribosomal antigen LcPO.

The United States Patent No. 6,525,186 by Bebate, *et al.*, in February 2003, is an isolated polynucleotide, comprising a recombinant cDNA encoding a chimeric polypeptide having 4 proteins LiP2a, LiP2b, LiH2a and LiPO of *Leishmania infantum* useful in serological diagnosis of canine leishmaniasis and protein obtained contains at least one antigenic determinant, recognized by serum from dogs with Visceral Leishmaniasis. In contrary, the applicant's invention provides methods and compounds, which deal with molecular diagnostic or Nucleic acid amplification based tests.

The United States Patent No. 6,613,337 by Reed, *et al.*, in September 2, 2003, deals with a fusion protein and a physiologically acceptable carrier, wherein the fusion protein comprises the amino acid sequence of SEQ ID NO: 24, for use in the therapy and diagnosis of leishmaniasis. The combination contains polypeptides that comprise immunogenic portions of M15, Ldp23, Lbhsp83, Lt-1 and LbeIF4A. However, the applicant's present invention does not deal with application of fusion protein.

The United States Patent No. 6,638,517 by Reed, *et al.*, in October 28, 2003, Leishmania antigens for use in the therapy and diagnosis of leishmaniasis teaches compositions and methods for preventing, treating and detecting leishmaniasis and stimulating immune responses in patients. The compounds provided include polypeptides that contain an immunogenic portion of one or more Leishmania antigens, or a variant thereof. The patent also discloses vaccines and pharmaceutical compositions comprising such polypeptides, or polynucleotides encoding such polypeptides, are also provided and may be used, for example, for the prevention and therapy of leishmaniasis, as well as for the detection of Leishmania infection.

United States Patent Application 20030162182, Salotra Poonam *et al.*, August 28, 2003, Species-specific PCR assay for detection of Leishmania donovani in clinical samples

of kala-azar and post kala-azar dermal leishmaniasis teaches methods and compounds for the polymerase chain reaction (PCR) assay for the diagnosis of leishmaniasis using specific novel oligonucleotide primers for the identification of *Leishmania donovani* parasites in clinical samples.

The applicant's invention uses a target in the genomic DNA of *leishmania* that is entirely different from the Salotra *et al.* (Us application No. 20030162182), work, where they amplify the minicircles in the kinetoplast DNA which is a type of mitochondrial DNA and very les in quantity, thereby providing less primer targets yielding to poor sensitivity. The present invention uses genomic DNA as a target for the PCR amplification which is in abundance in the parasite and thus better sensitvity. The DNA sequences amplified are from genomic DNA whereas Dr. Salotra method amplifies a region of the mitochondrial DNA, which is difficult to isolate as compared to genomic DNA and requires more expertise and facilities. Also the amount of mitochondrial DNA (K-DNA) isolated is much lower than the amount of genomic DNA isolated. The method developed by Salotra cannot differentiate between VL and PKDL forms of leishmania. So the present invention is more convenient to perform with having ability of differentiating the species more specifically.

The present invention provides a direct method of detecting Leishmania by amplification of the conserved repeat region of the kinesin-related gene, whereas all the other reported methods are based on the polypeptide derived from the kinesin-related gene (antigen-antibody tests). Another feature of this invention is that, the PCR method can differentiate between visceral Leishmaniasis (VL) and post kala-azar dermal leishmaniasis (PKDL).

OBJECTS OF THE INVENTION

The main object of the present invention is to develop novel oligonucleotide primers for amplification of the kinesin-related gene of *Leishmania* species. Further the object is to design primers based on the repetitive region of the kinesin-related gene for PCR amplification.

Another object of the invention is to develop a method for PCR amplification to detect Leishmaniasis in the patients infected with *Leishmania donovani* strains based on the conserved repeat region of kinesin-related gene of *Leishmania* species using novel oligonucleotide primers.

Another object of the present invention is to develop a method for detecting and differentiating VL and PKDL causing strains of leishmaniasis using the novel oligonucleotide primers.

Yet another object of the present invention is for a method of detection for leishmaniasis from a sample which is selected from either clinical samples or culture samples.

Further object of the present invention is to develop a diagnostic kit for detecting and differentiating the VL /PKDL strains/forms of the leishmaniasis, comprising of novel oligonucleotide primers, a reaction buffer, DNA polymerase, *Taq* polymerase.

BRIEF DESCRIPTION OF ACCOMPANYING DRAWING

Figure 1: PCR amplification of the DNA from various VL and PKDL causing strains of *Leishmania donovani*. The lanes depict the following: Lane A represents molecular weight marker, PKDL (Lane B, strain RMP-240; Lane C, strain RMP-142; Lane D, strain RMP-155; Lane E, strain RMP-19); and *Leishmania donovani* strains causing visceral diseases [Lane F, DD-8 (WHO reference strain); Lane G, strain RMR-1; Lane H, strain KE-16, Lane I, UR-6]; Lane J is a Clinical (Blood) sample of patient positive for visceral Leishmaniasis, Lane K, Healthy Human DNA Sample and Lane L, is a blood sample from a patient with CMV infection (Disease Control).

SUMMARY OF THE INVENTION

Accordingly, the present invention provides novel oligonucleotide primers for amplification of the kinesin-related gene of *Leishmania* species comprising of SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4, wherein the novel oligonucleotide primers are designed based on the tandem repeat region of the kinesin-related gene of *Leishmania* species.

The present invention also provides a method based on the amplification of the tandem repeat region of Kinesin-related gene from various strains of *L. donavani* using the primers (SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4) to detect and differentiate visceral leishmaniasis (VL) and post kala-azar-dermal leishmaniasis (PKDL) causing strains of leishmaniasis. The invention provides a method using multiplex PCR for detecting and differentiating visceral leishmaniasis (VL) and post kala-azar-dermal leishmaniasis (PKDL) causing strains of *Leishmania donovani* in a sample, comprising isolating DNA from a sample; amplifying the target region from the DNA using novel oligonucleotide primers and heat stable DNA polymerase to obtain amplified fragments; separating the amplified fragments and analyzing the fragments to detect and differentiate

VL and *PKDL* causing strains of *Leishmania donovani* based on the banding pattern of the amplified fragments. In addition, the present invention provides a diagnostic kit for detection and differentiation of VL and PKDL causing strains of the *Leishmania donovani.*

DETAILED DESCRIPTION OF THE INVENTION

In accordance, the present invention provides novel novel oligonucleotide primers for amplification of the kinesin-related gene of *Leishmania* species comprising of SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4.

An embodiment of the present invention provides for a method for detecting and differentiating visceral leishmaniasis (VL) and post kala-azar-dermal leishmaniasis (PKDL) causing strains of *Leishmania donovani*, the said method comprising the steps of:

a) isolating DNA from sample;

b) amplifying the target region from the DNA of step (a) using novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4 and heat stable DNA polymerase to obtain amplified fragments;

c) separating the amplified fragments of step (b); and

d) analyzing the fragments of step (c) to detect and differentiate *VL* and *PKDL* causing strains of *Leishmania donovani* based on the banding pattern of the amplified products.

In another embodiment of the present invention provides for a method wherein the sample for detection is selected from either clinical samples or culture samples.

Yet another embodiment of the present invention provides for a method wherein the sample for detection is selected from a group consisting of blood, bone marrow aspirate, bone marrow biopsy, splenic aspirate, splenic biopsy, liver aspirate, liver biopsy, lymph node aspirate, lymph node biopsy, skin scrapping, slit biopsy and other tissue materials.

Still another embodiment of the present invention provides for a method wherein the use of heat stable DNA polymerase preferably *Taq* polymerase and the amplification is carried out by polymerase chain reaction.

In another embodiment of the present invention provides for a method wherein separation of the amplified products is by gel electrophoresis and the detection of the amplified products is by ethidium bromide or other DNA stains.

Further embodiment of the present invention provides for a kit for detection and differentiation of VL and PKDL causing strains of the *Leishmania donovani*, comprising of novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4, reaction buffer, *Taq* polymerase, DNA marker, positive and negative control samples and instruction manual.

The present invention relates a method for detection of *Leishmaniasis* wherein the protozoan parasites of the genus *Leishmania* are the causative agents of visceral leishmaniasis (VL), also called kala-azar (KA). KA is a symptomatic infection of the liver, spleen and bone marrow caused by organisms of *Leishmania donovani* complex. PKDL (Post kala-azar dermal leishmaniasis) is an unusual dermatosis that develops as a sequel of KA. producing gross cutaneous lesions in the form of hypopigmented macules, erythema and nodules. The disease is relatively common in the Indian subcontinent and less frequent in East Africa, but exceptional in the American and European continents. Detection and characterization of *Leishmania* from patients of both KA and PKDL is important for deciding treatment regimens as well as for understanding the disease epidemiology. In many patients the dermal manifestations are seen even when the patient never had visceral form hence the term post-kala-azar dermal leishmaniasis is a misnomer. It is also seen that no kala-azar patient has ever developed PKDL once he/she has migrated to a PKDL non-endemic area after kala-azar treatment. Therefore, the applicant proposed a hypothesis that VL and PKDL causing strains of *Leishmania donovani* are different. To elucidate the proposed hypothesis, the applicant successfully designed and standardized an Alu-PCR and its primers to differentiate between these two strains.

The present invention provides a unique PCR amplification method to amplify the Kinesin-related gene of different Indian isolates of *Leishmania donovani.* This has been developed by the applicant to analyze genetic differences of the strains causing VL and PKDL on the basis of number and size of the bands as a result of PCR assay. The PCR assay for detection and differentiation between the strains of *Leishmania donovani* that cause visceral léishmaniasis and strains that cause post kala-azar-dermal leishmaniasis was developed using the following sets of PCR primers:

Forward Primer (SS-KIN 1): SEQ ID NO: 1

5' CTAGAGCAGCAGCTTCG 3' (17 oligomer)

Forward Primer (SS-KIN 3): SEQ ID NO: 2

5' CTTGAGCAGCAGCTTCG 3' (17 oligomer)

Reverse Primer (SS-KIN 2): SEQ ID NO: 3

5' CGTGGCCCTCGTGTTCT 3' (17 oligomer)

Reverse Primer (SS-KIN 4) SEQ ID NO: 4

5' CGCGGCCCTCGTGTCCT 3' (17 oligomer)

The invention further provides a method to differentiate VL and PKDL strains using an the above primers sets having SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, which are designed on the basis of consensus, repetitive 117bp sequences in the Kinesin-related gene of *Leishmania donovani* strain MHOM/IN/DD8. The present invention teaches improved methods for differentiation of VL and PKDL causing strains of *Leishmania donovani* based on the PCR amplification of the Kinesin-related gene .

The present invention is described below. Although methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. The materials, methods, and examples are illustrative only and are not intended to be limiting. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

GROWTH OF STRAINS

Parasites were initially isolated as Promastigotes in Novy-Mac Neal Nicolle (NNN) [Sundar, et al., 2002] medium from clinical samples of Kala-azar and post Kala-azar dermal leishmaniasis patients and subsequently adapted to grow at 25^0C in Medium 199 containing 10% heat inactivated FCS. For routine maintenance, samples of the inoculum containing parasites were introduced aseptically into culture tubes with 4ml of Medium-199 [Sundar, et al., 2002] supplemented with 10% FCS. The tubes were placed in cooled incubator at 25^0C and the growth was monitored at regular intervals by microscopy. For mass cultivation of the parasite, samples of inoculum containing parasites were introduced aseptically into 200ml of M199 containing 10%FCS in a 500ml tissue culture flask and incubated in a cooled incubator at 25^0C until mid log phase (7-10 days). The parasites were then harvested and used for nuclear DNA isolation.

STRAINS- CULTURE MAINTAINENCE

The VL and PKDL strains, as described herein were isolated from various parts of India (Table 1) and maintained in Medium 199 supplemented with 10% fetal calf serum and mass culture propagation for DNA isolation for PCR were done in medium 199 with 5%

FCS +5% human urine (post menopausal female) and culture flasks incubated with agitation at 17-20^{0}C in a BOD incubator

Table1:

Strain ID	Source	Geog. Location	Disease
1. HM/IN/DD8	WHO std. strain	Bihar	VL
2. HM/IN/UR6,	IICB, Calcutta	West Bengal	VL
3. HM/IN/Ag83,	IICB, Calcutta	West Bengal	VL
4. HM/IN/SS,	PGIMER, Chd.	Bihar	VL
5. HM/IN/LD183,	Our Lab, AIIMS	Bihar	VL
6. HM/IN/KE16,	Our Lab, AIIMS	Bihar	VL
7. HM/IN/J1,	Our Lab, AIIMS	Bihar	VL
8. HM/IN/J2,	Our Lab, AIIMS	Bihar	VL
9. HM/IN/J3,	Our Lab, AIIMS	Bihar	VL
10. HM/IN/RMRI,	RMRI, Patna	Bihar	PKDL
11. HM/IN/RMP7,	RMRI, Patna	Bihar	PKDL
12. HM/IN/RMP8,	RMRI, Patna	Bihar	PKDL
13. HM/IN/RMP142,	RMRI, Patna	Bihar	PKDL
14. HM/IN/RMP155,	RMRI, Patna	Bihar	PKDL
15. HM/IN/RMP240,	RMRI, Patna	Bihar	PKDL
16. HM/IN/RS,	IICB, Calcutta	Not known	VL
17. HM/IN/MF,	IICB, Calcutta	Not known	VL
18. HM/IN/GEI,	IICB, Calcutta	Not known	VL
19. HM/IN/GEIV	IICB, Calcutta	Not known	VL

DNA ISOLATION

The parasites in their mid log phase was harvested by centrifuging at 5000 rpm in a refrigerated centrifuge. Parasite nuclear DNA was isolated following standard protocol [Lu H.G. *et al.*, 1994] with minor modifications. Approximately 1-5 X 10^9 promastigotes were lysed in 10 volumes of lysis buffer (NaCl, 100 mM, Tris-HCl, 10mM (pH 8.0), EDTA 10mM, Proteinase K/ml 100μg, Sarcosyl 1.5%) at 60 ^{0}C for 3 hours. The kinetoplast DNA networks were sedimented by centrifugation at 27,000 X g for 1 hour and resuspended in TE buffer (Tris-HCl (pH 8.0) 10mM, EDTA (pH 8.0) 1mM). The nuclear DNA was

isolated from the supernatants left after sedimentation of the kinetoplast DNA. These supernatants were incubated overnight for further digestion of proteins at 65^0 C. The nuclear DNA was subjected to several cycles of phenol/chloroform extractions by adding equal volume of phenol/chloroform mixture, mixing thoroughly followed by sedimentation by centrifugation at 5000 rpm for 15 minutes. The nuclear DNA was precipitated by adding $1/10^{th}$ the volume of 3M-sodium acetate and 2 volumes of 100% ethanol mixed well and incubated at -20^0C for 1 hour. The mixture was sedimented by centrifugation at 5000 rpm for 30 minutes at 4^0C. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer. The concentration and purity of the DNA was measured by taking OD at 260/280nm.The DNA was also checked using agarose gel electrophoresis using standard DNA as marker for quantification. The DNA was stored at -70^0C until use.

The DNA from clinical samples is extracted by adding 300μl of patient whole blood to RBC or tissue lysis solution in 1.5ml Microfuge tube followed by mixing and incubation at room temperature for 30 minutes and treatment given with genomic DNA lysis solution, and centrifuged at 12,000 rpm for 5 minutes. carefully removed all but 30-50 μl of supernatant and to the supernatant add 200 μl of Instagene Matrix (Bio-Rad, USA) to the tube. after incubation at 56^0C for 30 minutes, the contents vortexed at high speed for 10 seconds, heated at 100^0 C in a heating block for 5 minutes, vortexed again and finally re-centrifuged at 12,000 rpm for 5 minutes, 20μl of the isolated DNA from the supernatant was taken for PCR.

PCR Assay

The PCR assay was standardized using the DNA isolated from the cultures of VL and PKDL causing strains of *Leishmania Donovan*. These cultures were maintained under laboratory conditions using conventional methods. The PCR assay was further carried out using the whole blood sample of *Leishmania donovani* patients for standardization. The PCR amplification was carried out using all the four novel oligonucleotide primers namely SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4. The details of the primers are given below.

Forward Primer (SS-KIN 1): SEQ ID NO: 1

5' CTAGAGCAGCAGCTTCG 3' (17 oligomer)

Forward Primer (SS-KIN 3): SEQ ID NO: 2

5' CTTGAGCAGCAGCTTCG 3' (17 oligomer)

Reverse Primer (SS-KIN 2): SEQ ID NO: 3

5' CGTGGCCCTCGTGTTCT 3' (17 oligomer)

Reverse Primer (SS-KIN 4) SEQ ID NO: 4

5' CGCGGCCCTCGTGTCCT 3' (17 oligomer)

The assay can be termed as multiplex PCR assay. The novel primers were designed based on the consensus repetitive 117 bp region of the Kinesin-related gene of *Leishmania donovani*. The PCR amplification method of the present invention was based on the data concerning structure and organization of repetitive elements in the human genome and having similarity to the sequences in the *Leishmania* species and hence is called as Alu-PCR [Piarrous R *et al.*, 1993]. It has been observed that the primers originating from repetitive sequences recognize and differentiate the locus in a specific manner on the basis of the size of the repetitive element present in the species, which varies in their sizes due to the intron sequences (non-coding regions).

The novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4 were designed based on the consensus, repetitive 117 bp sequence of the Kinesin-related gene of *Leishmania donovani* strain MHOM/IN/DD8. All the four primers (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4) were employed in the PCR assay for the detection of *Leishmaniasis* using multiplex PCR strategy.

PCR amplification of the kinesin-related gene of different Indian isolates of *L. Donovani* and clinical samples positive for *Leishmaniasis* (serologically positive) were carried out following the method exclusively developed by the applicant using the novel primers having SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4. These primers were uniquely designed based on the 117bp fragment of the kinesin-related gene to amplify the repeated region of the kinesin-related gene. The PCR amplified products were electrophoresed in 2.0% to 2.5% agarose gel and stained with ethidium bromide. The products were visualized under an UV-transilluminator (UVP) for identification of banding pattern.

The present invention teaches a method for detection of VL and PKDL causing strains of *Leishmania donovani* based on novel oligonucleotide primers designed for amplification of the kinesin-related gene. This method is based on multiplex PCR amplification and employs four primers (SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4) in a single reaction mixture. The PCR amplified products were resolved on gel electrophoresis and detected by standard methods. The banding patterns from the

various samples were analyzed for detection of VL and PKDL causing strains of *Leishmania donovani*.

The detailed procedure of the PCR assay is given below:

The PCR assay was carried out in 50 µl reaction mixture for the various samples for detection of VL and PKDL causing strains of *Leishmania donovani*.

Each 50 µl reaction mixture contain 100 ng of nuclear DNA (isolated from samples), 200 uM each of deoxyribonucleotide triphosphates (dNTPs), 1.5 units of Taq DNA Polymerase, 5 ul of 10 X PCR buffer (100 mM TAPS (pH 8.8), 15 mM MgCl2, 500 mM KCl and 0.1% gelatin). The reaction mixture was incubated at temperature of 94°C for 5 min before starting the PCR amplification cycles. The temperatures used for amplification cycles were 94°C for 60s, 52°C for 60s, 72°C for 60s. This was carried out for 25-36 cycles followed by 72°C for 10 min for extension. The PCR amplified products were electrophoresed in 2.0% to 2.5% agarose gels, stained with ethidium bromide and visualized on an UV-transilluminator to detect the banding pattern of the products from the various samples assayed.

The banding pattern of the amplified DNA products was different for VL and PKDL causing strains as observed in Figure 1. A ladder banding pattern was obtained for both the VL and PKDL strains however the banding pattern was different for both these strains. The number of amplified products varied from 8-10 for VL causing strains whereas it was 6-8 bands in PKDL causing strains as seen in Figure 1.

All the four primers were used for amplification at equimolar concentration in a single PCR reaction mixture. It has been observed that primers originating from repetitive sequences recognize a locus in a specific manner likewise the primers SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4 bind in strain specific manner. The amplified products showed distinct ladder pattern of bands for VL and PKDL causing strains, in which the number of bands for VL strains were 8-10 whereas for PKDL strains 6-8 bands were detected. Most of the PCR amplified products were in the size range of 0.2-2.0 kb after PCR amplification from VL strains while in the case of PKDL strains the amplified products were in the range of 0.2 to 1.2 kb in length.

The ladder of amplified products showed distinct pattern of banding for VL strains and PKDL strains, in which the number of bands for VL strains were 8-10 and for PKDL strains 6-8. A representative banding pattern of PCR amplified products is depicted in Figure 1. The figure 1 clearly shows differences in banding pattern between the PKDL and

VL causing strains. Based on the banding pattern of the amplified products the *Leishmania* strains from kala-azar (visceral leishmaniasis,VL) patients showed more bands as compared to bands amplified from PKDL causing strains. Bands were amplified in the range 0.2 to 2.0 kb in VL strains which are namely of size 2.0kb, 1.4kb, 1.0kb, 0.7kb, 0.6kb, 0.45 kb and 0.4kb. Faint bands were also detected at around 0.2kb. An intense signal was observed in all these lanes (VL) around 0.45-0.4 kb. This may be due to the presence of doublet bands. This prominent band of 0.45 kb size was absent from PKDL strains (Fig 1).

In the case of PKDL (dermal leishmaniasis of Bihar) causing strains, the number of PCR bands was significantly less. The number was between 6-8 bands. All the PCR amplified products (seen as bands in the fig 1) were observed in the range of 0.20-1.2 kb. Among this the most prominent were bands of sizes 1.2Kb, 0.85Kb, 0.8K, 0.6 KB, 0.4 KB and 0.36 bands. A faint band at around 0.2Kb was observed in PKDL strains (Fig 1). The band at 0.85 showed intense signal and may be a doublet of two bands of sizes 0.85 and 0.8 Kb. Significantly this prominent band was absent in VL strains. In addition , negative controls were included in the PCR assay using the same primers and PCR conditions. These are represented in Figure 1 (see lanes K and L). The controls are healthy human DNA sample (Lane K) and human DNA sample infected with CMV (Lane L).Av smear was obtained as excess DNA amount (template) was taken for the analysis.

After disclosing the primers to differentiate the two causative strains of *Leishmania*, the applicant found that dermal manifestations of *Leishmaniasis* in Bihar and adjoining areas are due to *in-vivo* hybridization and development of quasi species. This invention will help in identifying the organism and its associated disease, whether it will cause VL and PKDL form when the source of isolation is not known. This invention will also help in identifying the specific strain and to trace the source of infection (reservoir) the issue, which has remained unresolved in India till date.

These results, also suggests that VL and PKDL causing agents are genetically different. The following factors need consideration namely.

1.) PKDL being considered to be the sequel of infection with *Leishmania donovani*

2.) The presence of kinesin-related gene conserved only in visceralising species but minor genotypic differences between VL and PKDL isolates imply that, PKDL may be due to recombination between the two *Leishmanial species* co-infecting the same host and then evolving a new strain causing PKDL.

The use of multiplex PCR using SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4 primers is for detection of *Leishmania donovani* infection and to

differentiate the strains whether these will cause visceral form of Leishmaniasis (kala-azar) or a dermal form of Leishmaniasis in Bihar commonly known as PKDL.

The invention is further illustrated with the following examples and these examples are not to limit the scope of the invention.

EXAMPLES

Example 1

Growth of Strains: Parasites were initially isolated as Promastigotes in NNN medium from clinical samples of Kala-azar and post Kala-azar dermal leishmaniasis patients and subsequently adapted to grow at 25^0C in Medium 199 containing 10% heat inactivated FCS. For routine maintenance, samples of the inoculum containing parasites were introduced aseptically into culture tubes with 4ml of medium 199 supplemented with 10% FCS. The tubes were placed in cooled incubator at 25^0C and the growth was monitored at regular intervals by microscopy. For mass cultivation of the parasite, samples of inoculum containing parasites were introduced aseptically into 200ml of M199 containing 10%FCS in a 500ml tissue culture flask and incubated in a cooled incubator at 25^0C until mid log phase (7-10 days). The parasites were then harvested and used for nuclear DNA isolation

Example 2:

DNA Isolation: The parasites in their mid log phase was harvested by centrifuging at 5000 rpm in a refrigerated centrifuge. Parasite nuclear DNA was isolated following standard protocol [Lu H.G. *et al.*, 1994] with minor modifications. Approximately 1-5 X 10^9 promastigotes were lysed in 10 volumes of lysis buffer (NaCl, 100 mM, Tris-HCl, 10mM (pH 8.0), EDTA 10mM, Proteinase K/ml 100μg, Sarcosyl 1.5%) at 60^0 C for 3 hours. The kinetoplast DNA networks were sedimented by centrifugation at 27,000 X g for 1 hour and resuspended in TE buffer (Tris-HCl (pH 8.0) 10mM, EDTA (pH 8.0) 1mM). The nuclear DNA was isolated from the supernatants left after sedimentation of the kinetoplast DNA. These supernatants were incubated overnight for further digestion of proteins at 65^0 C. The nuclear DNA was subjected to several cycles of phenol/chloroform extractions by adding equal volume of phenol/chloroform mixture, mixing thoroughly followed by sedimentation by centrifugation at 5000 rpm for 15 minutes. The nuclear DNA was precipitated by adding $1/10^{th}$ the volume of 3M-sodium acetate and 2 volumes of 100% ethanol mixed well and incubated at -20^0C for 1 hour. The mixture was sedimented by centrifugation at 5000 rpm for 30 minutes at 4^0C. The pellet was washed with 70% ethanol, dried and resuspended in

TE buffer. The concentration and purity of the DNA was measured by taking OD at 260/280nm.The DNA was stored at -70^{0}C until use.

Example 3

PCR assay for Leishmaniasis:

The PCR assay was carried out using the DNA isolated (Example 2 gives details for DNA isolation) from the various strains as given in Table 1. The PCR amplification was carried out using all the four novel oligonucleotide primers namely SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4. . The novel primers were designed based on the consensus repetitive 117 bp region of the Kinesin-related gene of *Leishmania donovani*. The PCR assay can also be termed as multiplex PCR. The details of the primers are given below.

Forward Primer (SS-KIN 1): SEQ ID NO: 1

5' CTAGAGCAGCAGCTTCG 3' (17 oligomer)

Forward Primer (SS-KIN 3): SEQ ID NO: 2

5' CTTGAGCAGCAGCTTCG 3' (17 oligomer)

Reverse Primer (SS-KIN 2): SEQ ID NO: 3

5' CGTGGCCCTCGTGTTCT 3' (17 oligomer)

Reverse Primer (SS-KIN 4) SEQ ID NO: 4

5' CGCGGCCCTCGTGTCCT 3' (17 oligomer)

Each 50 µl reaction mixture contains 100 ng of nuclear DNA (isolated from samples), 200 uM each of deoxyribonucleotide triphosphates (dNTPs), 1.5 units of Taq DNA Polymerase, 5 ul of 10 X PCR buffer (100 mM TAPS (pH 8.8), 15 mM MgCl2, 500 mM KCl and 0.1% gelatin). The reaction mixture was incubated at temperature of 94°C for 5 min before starting the PCR amplification cycles. The temperatures used for amplification cycles were 94°C for 60s, 52°C for 60s, 72°C for 60s. This was carried out for 35-36 cycles followed by 72°C for 10 min for extension. The PCR amplified products were electrophoresed in 2%. to 2.5% agarose gels, stained with Ethidium Bromide and visualized on an UV-transilluminator to detect the banding pattern of the products from the various samples assayed.

All the four primers were used for amplification at equimolar concentration in a single multiplex -PCR reaction mixture. It has been observed that under certain conditions,

primers originating from repetitive sequences recognize a locus in a specific manner; likewise the primers SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4 bind in strain specific manner. The ladder of amplified products showed distinct pattern of bands for VL strains and PKDL strains, in which the number of bands for VL strains were 8-10 and for PKDL strains 6-8 bands were detected. Most of the PCR amplified products were in the size range of 0.2-2.0 kb from VL strains while in PKDL strains the bands ranged from 0.20 1.2 kb. These are shown in Figure1 and the details are given in the description above. Based on the banding pattern after amplification, it is clear that VL and PKDL causing strains of *Leishmania donovani* can be differentiated. From Fig.1, it is clear that 2 bands of molecular weight 1.4 Kb and 2.0 Kb are amplified in samples which are VL causing strains but are absent in PKDL causing strains of *Leishmania donovani*. An other important differencd observed in Fig. 1 is the presence of a prominent band with strong signal in VL strains around 0.45 Kb , which is absent in PKDL strains. Simiarly, an prominent band with strong signal around 0.8 Kb is present in PKDL strains which is absent in VL strains. These differences in banding pattern can be used to differentiate between VL and PKDL causing strains of *Leishmania donovani.*

Example 4

Direct PCR Assay:

The DNA from clinical samples is extracted by adding 25µl of patient whole blood to 1 ml of sterile distilled water in 1.5ml Microfuge tube followed by mixing and incubation at room temperature for 30 minutes, centrifuged at 12,000 rpm for 5 minutes, carefully removed all but 30-50 µl of supernatant and added 200 µl of Instagene Matrix (Bio-Rad, USA) to the tube, after incubation at 56^0C for 30 minutes, the contents vortexed at high speed for 10 seconds, heated at 100^0 C in a heating block for 5 minutes, vortexed again and finally re-centrifuged at 12,000 rpm for 5 minutes, 20µl of the isolated DNA from the supernatant was taken for PCR. Further, in the experiment 100-150 ng of nuclear DNA from different isolates were amplified for 36 cycles in 50 µl reaction mixtures, each containing, 200 µM each of deoxynucleoside triphosphates (dNTPs). 1.5 units of *Taq DNA Polymerase* (Perkin Elmer) and 5 µl of 10 X PCR buffer (100 mM TAPS (pH 8.8), 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin). The working concentration of each primer was 0.5 µM. The temperature cycles used were: 94 ^{0}C for 10 min; 94 ^{0}C for 60s, 52 ^{0}C for 60s, 72 ^{0}C for 60s followed by 72 ^{0}C for 10 min. The PCR products were electrophoresed in 1.5%-2.5% agarose gels, stained with Ethidium Bromide and visualised on an UV-

transilluminator. The data obtained from this assay was similar to the data obtained as shown in Example 3. The amplified products showed distinct ladder pattern of bands for VL and PKDL causing strains, in which the number of bands for VL strains were 8-10 whereas for PKDL strains 6-8 were detected. Most of the PCR amplified products were in the size range of 0.2-2.0 kb after PCR amplification from VL strains while in the case of PKDL strains the amplified products were in the range of 0.2 to 1.2 kb in length.

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I / We claims

1. Novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4 for amplification of the kinesin-related gene of *Leishmania* species.

2. A method for detecting and differentiating visceral leishmaniasis (VL) and post kala-azar-dermal leishmaniasis (PKDL) causing strains of *Leishmania donovani* in a sample, the said method comprising the steps of :

   a) isolating DNA from a sample;

   b) amplifying the target region from the DNA of step (a) using novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4, and heat stable DNA polymerase to obtain amplified fragments;

   c) separating the amplified fragments of step (b); and

   d) analyzing the fragments of step (c) to detect and differentiate *VL* and *PKDL* causing strains of *Leishmania donovani* based on the banding pattern of the amplified fragments.

3. A method of claim 2, wherein in step (a) the sample is either clinical sample or culture sample.

4. A method of claim 3, wherein the clinical sample is selected from a group consisting of blood, bone marrow aspirate, bone marrow biopsy, splenic aspirate, splenic biopsy, liver aspirate, liver biopsy, lymph node aspirate, lymph node biopsy, skin scrapping, slit biopsy and other tissue materials.

5. A method as claimed in 2 wherein in step (b) the heat stable DNA polymerase is *Taq polymerase.*

6. A method as claimed in 2 wherein in step (b) the amplification is done by polymerase chain reaction.

7. A method as claimed in 2 wherein in step (c) the separation is done preferably by gel electrophoresis.

8. A method as claimed in 2 wherein in step (d) the detection is by ethidium bromide or other DNA stains.

9. A diagnostic kit for detection and differentiation of VL and PKDL causing strains of the *Leishmania donovani*, comprising of novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4, reaction buffer, *Taq polymerase*, DNA marker, positive and negative control samples and instruction manual.

10. A method for detecting and differentiating VL and PKDL causing strains of *Leishmania donovani* as herein described with reference to examples and figures.

DATED THIS 22ND DAY OF DECEMBER 2004

T.SRINIVASAN
AGENT FOR THE APPLICANTS

TO
THE CONTROLLER OF PATENTS
PATENT OFFICE AT NEW DELHI

22 DEC 2004

Abstract

The present invention relates to a novel oligonucleotide primers having SEQ ID NO: 1, SEQ ID No: 2, SEQ ID NO: 3 and SEQ ID NO: 4 for amplification of the kinesin-related gene of *Leishmania* species. The invention also provides a method for detecting and differentiating visceral leishmaniasis (VL) and post kala-azar-dermal leishmaniasis (PKDL) causing strains of *Leishmania donovani* in a sample, comprising isolating DNA from a sample; amplifying the target region from the DNA using novel oligonucleotide primers and heat stable DNA polymerase to obtain amplified fragments; separating the amplified fragments and analyzing the fragments to detect and differentiate *VL* and *PKDL* causing strains of *Leishmania donovani* based on the banding pattern of the amplified fragments. In addition, the invention provides a diagnostic kit for detection and differentiation of VL and PKDL causing strains of the *Leishmania donovani*.

Applicant Name: **All India Institute of medical sciences** 22 DEC 2004

Application No:

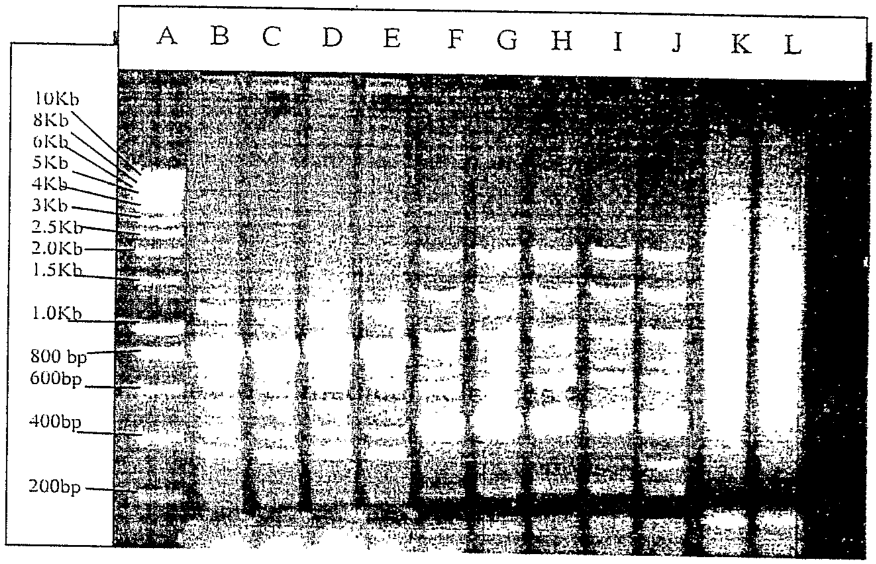

Figure 1

T.Srinivasan

Agent for the Applicants

PCT/IN2004/000395